



BACKGROUND

1
2
3 This invention relates to the formation of stable branch
4 migration structures and the various applications of these structures.
5

6 The displacement of one strand of a double-stranded nucleic
7 acid by another single strand with an identical nucleotide sequence is
8 a well documented aspect of DNA or RNA replication and genetic
9 recombination *in vivo*. Branch points are found in nucleic acids that
10 are undergoing this kind of strand displacement where two strands
11 compete for base pairing interactions with complementary sequences
12 of a third strand. The movement of branch points along the strands of
13 the nucleic acid, branch migration, does not require the action of
14 specific enzymes or proteins.
15

16 The phenomenon of branch migration *in vitro* in the renatured
17 molecules of terminally repetitious, circularly permuted bacteriophage
18 DNA was first reported by Lee, Davis, and Davidson [JMB 48: 1-22
19 (1970)]. Branched nucleic acid structures suitable for the study of
20 strand displacements can be constructed *in vitro* using various
21 hybridization conditions.
22

23 Branch migration has been exploited to form or resolve DNA-
24 RNA hybrids. In solutions without formamide, a DNA strand will
25 displace RNA from a DNA-RNA hybrid. This reaction is the basis of a
26 homogeneous nucleic acid hybridization assay developed by Vary *et al.*
27 of Allied Corporation [Nuc. Acids Res. (1987) 15, 6883-6897 and U.S.
28 Pat. 4,795,701]. This assay involves RNase digestion of the displaced
29 RNA strand, conversion of AMP to ATP and detection of the product of
30 the conversion by chemiluminescence using luciferase. Vary's method
31 is not applicable to DNA cloning.
32

33 In concentrated formamide solutions, a DNA strand may be
34 displaced by RNA to form an R-loop [see Thomas, M., White, R.L., &
35 Davis, R.W. (1976) Proc. Nat. Acad. Sci., USA 73, 2294-2298].
36 Conceptually, R-loop formation is analogous to the displacement of one

1 DNA strand from the end of a duplex. Regions of double-stranded
2 DNA can take up complementary RNA sequences to form R-loops
3 under conditions where RNA:DNA hybrids are more stable [Casey, J.
4 and Davidson, N. (1977) NAR 4: 1539-1552]. The enrichment of
5 specific DNA sequences has been accomplished using buoyant density
6 sedimentation to select for R-loop structures containing these
7 sequences. The technique of R-loop formation has not been patented.
8 To date, applications of R-looping procedures involve partial
9 denaturation of the target DNA and have not yielded products that can
10 be directly cloned into standard cloning vectors.

11
12 D-loop formation that is analogous to R-loop formation can occur
13 between a DNA strand and a superhelical DNA duplex [Radding, C.M.,
14 Beattie, K.L., Holloman, W.K., & Wiegand, R.C. (1977) J. Mol. Biol. 116,
15 825-839]. This reaction depends upon the superhelical free energy
16 and will, thus, not take place with linear DNA molecules. No cloning
17 technology based on this observation has been described. D loop
18 formation in superhelical DNA has been used for specific cleavage
19 [Corey, D.R., Pei, D. & Schultz, P.G. (1989) J. Am. Chem. Soc. 111,
20 8523-8525].

21
22 A method has been developed which uses RecA-coated strands
23 to overcome the limitation of D-loop formation to superhelical DNA
24 molecules [Rigas, B., Welcher, A.A., Ward, D.C., & Weissman, S.M.
25 (1986) Proc. Natl. Acad. Sci., USA 83, 9591-9595]. Labeling of the
26 single-stranded probe with biotinylated nucleotides facilitates
27 purification of the D-loop products of this reaction by affinity
28 chromatography. DNA hybrids that contain biotinylated nucleotides
29 have lower melting temperatures than unmodified DNA hybrids, i.e.,
30 biotin has a destabilizing effect on the helix [Langer, et al. (1981)
31 Proc. Natl. Acad. Sci. USA 78: 6633-6637]. In this method D-loop
32 formation requires implementation of a pretreatment step to facilitate
33 D-loop formation. Moreover, this procedure does not result in
34 products that are directly clonable into existing DNA cloning vectors.

1 A short DNA strand hybridized to a longer DNA strand will also
2 be rapidly displaced by a homologous, but longer, overlapping strand
3 *in vitro* [see Green, C. and Tibbetts, C. (1981) Nuc. Acids Res. 9,
4 1905-1918]. This observation forms the basis of diagnostic assays for
5 DNA or RNA sequences that are based on branch migration and DNA
6 strand displacement, described in Collins et al. [Mol. & Cell. Probes 2:
7 15-30 (1988)], Vary et al. [Clin. Chem. 32: 1696-1701 (1986)], U.S.
8 Patents 4,766,064 [Williams et al. (1988), Allied Corp.], 4,766,062
9 [Diamond et al (1988), Allied Corp.], and 4,795,701 [Vary et al (1988),
10 Allied Corp.] and European Patents 0167238 A1 [Collins et al (1985),
11 Allied Corp.] and 0164876 A1 (Collins et al. (1985) Allied Corp.).
12

13 In these assays, a partially double-stranded probe complex is prepared
14 with a detectable label on one of the two strands. This probe complex
15 is then incubated with a sample containing target nucleic acids (*i.e.*,
16 double-stranded nucleic acids that are at least partially homologous to
17 the single-stranded portion of the probe complex) under appropriate
18 hybridization conditions. The target nucleic acids hybridize to the
19 single-stranded portion of the probe complex and undergo branch
20 migration to release the labeled probe strand. The amount of labeled
21 strand released is proportional to the amount of target DNA in a
22 sample. Thus, this assay involves the use of a pre-formed partially
23 double-stranded probe complex to promote branch migration and
24 relies upon the transient nature of the branch migrated structure and
25 the total release of the labeled probe strand.
26

27 In these assays, the efficiency of the strand displacement reaction
28 could be enhanced by the addition of volume excluding reagents, such
29 as polyethers, or by pretreatment of the target with Rec A proteins.
30 Others have also noted that the Rec A protein promotes branch
31 migration that proceeds unidirectionally in the 3'→5' direction [Cox,
32 M.M. & Lehman, I.R. (1981) Proc. Natl. Acad. Sci. USA 78: 6018-
33 6022]. The diagnostic assays developed by the Allied Corp. use pre-
34 formed duplexes to promote displacement and do not relate to the
35 development of genetic engineering techniques or to the stabilization
36 of the branch migration structure.

1
2 Thus, the phenomenon of branch migration initiated by the
3 formation of a stable hybrid has been described in the literature.
4 Although the formation of branched or looped structures has been
5 used for identification, purification, and enrichment of DNA
6 sequences, this technique has not been applied to the development of
7 a directly clonable product. Moreover, stabilization of branch
8 migration structures would enhance the efficiency of procedures that
9 involve collection or identification of these entities. Simple methods
10 for preparing stable branch migration structures have not been
11 reported by others.

12
13 Experiments described more than 20 years ago showed that the
14 substitution of bromine at position C5 of pyrimidines leads to
15 increased duplex stability [Michelson et al.(1967) Prog. Nuc. Acid Res.
16 & Mol. Bio. 6: 84-141]. Radding et al. [J. Biol. Chem. 116: 2869-2876
17 (1962)] showed that dG-BrdC is a more thermally stable base pair than
18 dG-dC. In another study, poly dl:poly BrdC had a melting temperature
19 26°C higher than poly dl:poly dC [Inman & Baldwin (1964) J. Mol. Bio.
20 8: 452-469], and it was further shown that poly BrdC displaced poly
21 dC from a poly dl:poly dC duplex to form a new duplex with poly dl
22 [Inman J. Mol. Bio. 9: 624-637 (1964)]. These observations have not
23 been applied to the stabilization of branch migration structures by
24 displacer strands containing modified nucleotide bases.

25
26 Tatsumi and Strauss [Nuc. Acids Res. 5: 331-347 (1978)]
27 labeled DNA with bromodeoxyuridine (BrdUrd) *in vivo* in human
28 lymphoid cells and observed a high degree of branch migration after
29 isolation and shearing of the DNA. These workers suggested that this
30 high level of branch migration reflected the increased stability of
31 helices containing BrdUrd and the trapping of the branch migration
32 configuration. Their results further suggest that once formed,
33 halogen-substituted branch migration structures are relatively stable.
34 Tatsumi and Strauss did not investigate the phenomenon of branch
35 migration *in vitro*, did not use synthetic oligo- or polynucleotides
36 labeled with halogenated nucleotides, *i.e.*, pre-modified displacer

1 strands, in their experiments, and could not use the branch migration
2 structures resulting from their experiments for purposes of cloning or
3 mutagenesis.

4
5 Currently, specific DNA fragments derived from genomic DNA
6 are usually identified using Southern blot analysis of the restriction
7 enzyme digestion products of that genomic DNA. Southern blot
8 analysis of DNA is a multi-step procedure that generally involves the
9 use of radioisotopes and autoradiography for fragment identification
10 following electrophoresis, transfer of the electrophoresed products
11 to a nitrocellulose or nylon membrane, and hybridization of the
12 transferred products to sequence-specific DNA probes. Procedures
13 that provide for simultaneous labeling and identification of specific
14 DNA fragments would be significantly simpler, faster, and cheaper
15 than Southern blot analyses and would have a significant impact on the
16 field of genetic engineering.

17
18 Labeling of DNA sequences has come to imply the incorporation
19 of modified nucleotides at internal or terminal positions. Often this is
20 accomplished enzymatically using nucleotides labeled with small
21 compounds such as biotin, sulfhydryl groups, mercury, allylamine, and
22 digoxigenin. Enrichment or purification of these labeled nucleic acids
23 can be accomplished by affinity chromatography. For example,
24 biotinylated DNA, including D loops, can be selectively bound and
25 eluted from solid matrices bearing avidin or streptavidin groups, as
26 has been reported by Ward and his co-workers [Langer, *op. cit.*].
27 Similarly, DNA labeled with sulfhydryls can be purified by affinity
28 chromatography on mercurated agarose and mercurated DNA can be
29 purified based on its affinity for sulfhydryl groups. The enrichment of
30 mRNA from total RNA populations can be accomplished based on the
31 affinity of the polyA tails on the messengers for oligo-dT matrices.
32 Following their purification, these enriched or purified nucleic acid
33 sequences are often further subjected to a series of procedures that
34 render them clonable. Labeling procedures that permit sequence
35 enrichment or purification by affinity chromatography and then the
36

1 direct cloning of these enriched or purified fractions would have
2 significant advantages over existing techniques.

3
4 The identification or enrichment of a specific subset of
5 nucleotide sequences or of DNA fragments in isolated genomic DNA or
6 total populations of RNA for the express purpose of cloning those
7 sequences or fragments has been accomplished by a variety of
8 methods that include, but are not limited to those involving the
9 selection of DNA fragments capable of undergoing branch migration,
10 R-loop, or D-loop formation. Selective cloning of fragments in a
11 population has been achieved based on restriction endonuclease
12 cleavage sites, especially those that recognize a unique site [Brown,
13 N.L. & Smith, M. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3213-3216].
14 and size [Thomas, M., Cameron, J.R. & Davis, R.W. (1974) Proc. Natl.
15 Acad. Sci. U.S.A. 71, 4579-4583]. Although these strategies have been
16 used widely and successfully to clone a wide variety of genes, they are
17 not universal and have limited specificity.

18
19 Another application of genetic engineering techniques relates to
20 the modification of genetic material so as to add or delete nucleotide
21 bases, such as for therapeutic purposes. Efforts to either replace,
22 inactivate, or modify genetic material are currently in progress. In
23 higher eukaryotic organisms, the approaches taken have, thus far,
24 required that the agents used to bring about these changes be
25 incorporated into various plasmid vectors that contain all or part of
26 various viral genomes. Site-directed gene replacement in lower
27 eukaryotes, such as yeasts, has been accomplished. A serious
28 deterrent to the therapeutic manipulation of human genetic material
29 *in vivo* relates to the lack of a suitable, benign vector system. The
30 ability to perform targeted delivery and incorporation of genetic
31 material into chromosomal DNA without using a viral vector would
32 represent a major advance in the field of gene therapy.

33
34 Green and Tibbetts [*op. cit.*] expressed an interest in using
35 branched DNA structures for *in vitro* site-directed mutagenesis and, in
36 fact, were able to use stable D-loop structures in superhelical DNA for

1 target deletion mutagenesis [Proc. Natl. Acad. Sci. USA 77: 2455-2459
2 (1980)]. They were unable to achieve this goal with linear target DNA
3 molecules using the branch migrated structures they obtained from *in*
4 *vivo* labeling of cells with BrdUrd (see Green and Tibbetts, 1981, op.
5 cit.) due to the short half-lives of their branched structures.
6

7 Site-specific genetic manipulation has been described [Capecchi,
8 M.R. (1989) Science 244, 1288-1292] where a small proportion of the
9 DNA which becomes integrated into the host genome is directed, by
10 homologous recombination, to the desired target DNA. Unfortunately,
11 the additional integration events occur at random and may inactivate
12 or activate genes leading to deleterious consequences. Sequences of
13 DNA capable of initiating triple-helix formation have been reported.
14 [Francois, J.C., Saison-Behmoaras, T., Thoung, N.T. & Helene, V.
15 (1989) Biochemistry 28, 9617-9619; Povsic, T.J. & Dervan, P.B.
16 (1989) J. Am. Chem. Soc. 111, 3059-3061]. The utilization of triple
17 helix formation as an adjunct to site-specific genetic manipulation is
18 unknown in the art.
19

20 SUMMARY

21
22 In its most general terms our invention relates to techniques which
23 increase the probability of certain types of reactions proceeding in a
24 desired manner. Our techniques promote specificity of reaction and
25 provide
26

27 simple methods for preparing stable branch migration
28 structures.
29

30 procedures for simultaneous labeling and identification of
31 specific DNA fragments that are significantly simpler, faster, and
32 cheaper than Southern blot analyses.
33

34 labeling procedures that permit sequence enrichment or
35 purification by affinity chromatography and selective cloning.
36

1 a method of performing targeted delivery and incorporation of
2 genetic material into chromosomal DNA without using a viral
3 vector.
4

5 Branch migration is the process by which a single strand of nucleic
6 acid is inserted and replaces at least a portion of one strand of a
7 nucleic acid duplex. Branch migration is a useful technique for the
8 sequence-dependent attachment (capture) of an oligodeoxynucleotide
9 duplex containing a single-stranded tail into the end of a
10 deoxynucleotide molecule, or of the sequence dependent
11 incorporation of an oligodeoxynucleotide into a deoxynucleotide
12 molecule at a location other than at its end. Existing methods require
13 the formation of a stable hybrid prior to the initiation of branch
14 migration; our process allows initiation and formation of a branch
15 migrated complex without prior stabilization. We have achieved this
16 result by stabilizing the resulting branch migrated complex
17 concurrently with formation or thereafter, or both.
18

19 Our specific attachment procedure may be used (A) to label a
20 particular fragment for detection without blotting and subsequent
21 hybridization, (B) to mark a particular fragment for affinity
22 chromatography, (C) to facilitate cloning by introducing a new 5' or 3'
23 overhang compatible with a restriction endonuclease site in a cloning
24 vector, or (D) for other purposes which will become apparent from the
25 present disclosure.
26

27 The various methods and materials of our invention require a displacer
28 entity which is at least partially complementary to and is capable of
29 binding to a target. Both the displacer and target are oligo- or
30 polynucleotide sequences, either synthetic or naturally occurring. Our
31 novel displacer may be used as a single stranded entity in certain
32 embodiments of our invention; in other embodiments it is utilized as a
33 partially double stranded entity hybridized to a linker strand.
34

35 One embodiment of our invention provides a novel displacer-linker
36 duplex and an improved method of attaching a deoxynucleotide

1 displacer sequence to a strand of a target deoxynucleotide duplex.
2 The oligo- or polydeoxynucleotide displacer-linker duplexes of our
3 invention consist of two strands, a displacer strand and a linker
4 strand. The displacer strand contains a sequence of nucleotides at
5 least partially complementary to the linker strand and a sequence at
6 least partially complementary to one strand of a recipient
7 polydeoxynucleotide duplex.

8
9 The sequence-dependent attachment (capture) of an
10 oligodeoxynucleotide duplex containing a single-stranded tail can be
11 influenced by branch migration into the end of a DNA molecule. Our
12 novel oligo- or polydeoxynucleotide displacer-linker duplex is capable
13 of initiating branch migration at the end of a recipient
14 polydeoxynucleotide duplex without the prior formation of a stable
15 hybrid with such recipient polydeoxynucleotide duplex. More
16 particularly, these novel duplexes can hybridize to and initiate branch
17 migration at a restriction endonuclease cleavage site, in particular
18 adjacent to a 3' or 5' single stranded extension on a recipient
19 polydeoxynucleotide duplex.

20
21 Substitution of one or more of the nucleotides in the portion of the
22 displacer strand which is complementary to the recipient increases
23 DNA-DNA hybrid stability. Oligonucleotides containing the modified
24 nucleotide displace non-modified nucleotide containing strands from
25 the ends of duplexes. In the case of 3' or 5' overhangs the rate of
26 displacement is of the same order of magnitude as the nucleation
27 reaction of DNA reassociation.

28
29 We also provide a displacer which is not hybridized to a linker strand
30 and which is capable of initiating triple helix formation. This class of
31 displacers comprises

- 32 1. a first sequence which is capable of initiating triple helix formation,
33 and which comprises
 - 34 a) at least six consecutive pyrimidine bases or
 - 35 b) at least seven bases where at least six of the bases are pyrimidine
36 bases and the seventh base is guanine, and

- 1 2. a second sequence proximate to such first sequence which is
2 a) complementary to and runs antiparallel to the second strand of the
3 recipient duplex and
4 b) which is capable of initiating branch migration proximate to the
5 triple helix.
6

7 We further disclose displacers which are nuclease resistant and the
8 method of modifying a recipient duplex to confer nuclease resistance
9 to the duplex. These displacers contain at least one moiety attached
10 to a terminus of the oligo- or polynucleotide, which moiety confers
11 endonuclease resistance to the terminus to which it is attached.
12

13 We have found it desirable to modify at least one of the nucleotides in
14 the displacer strand which is at least partially complementary to one
15 strand of the recipient polydeoxynucleotide duplex. The nucleotide is
16 modified in a manner that increases the stability of the hybrid
17 displacer-recipient duplex.
18

19 In addition to stability increasing modifications, we have found it
20 useful to incorporate modified nucleotides in the displacer or linker
21 which permit detection of the displacer-recipient hybrid or its
22 isolation by affinity chromatography.
23

24 Another aspect of our invention is the hybrid structure formed when
25 the displacer or displacer-linker duplex is attached to the target.
26 Where the hybrid is the result of the attachment of the target to our
27 novel displacer-linker duplex, the linker strand is preferably
28 covalently linked to one of the strands of the recipient duplex.
29

30 In a preferred version of our invention, the hybrid structure
31 containing the attached displacer sequence of single stranded
32 deoxynucleotide is stabilized, most desirably by the presence of at
33 least one modified nucleotide in the displacer strand.
34
35
36

1 We also disclose a labelled hybrid structure. This labelled hybrid
2 structure incorporating our displacer-linker is useful in many
3 biochemical procedures, such as, for example, to facilitate capture of
4 the displacer-recipient hybrid by affinity chromatography, to label one
5 end of an cloned deoxynucleotide insert in a vector, to facilitate
6 restriction endonuclease mapping of an insert, to facilitate selectively
7 cloning a recipient polynucleotide duplex and to facilitate isolation of
8 clones of contiguous polydeoxyribonucleotides. The hybrid
9 incorporating the displacer is useful in affinity chromatography and to
10 facilitate 1] detection of specific oligo- or polynucleotides and 2] site
11 specific genetic manipulation

12
13 Our methods involve the stabilization of a non stable complex between
14 one strand of a recipient polydeoxynucleotide sequence and a
15 displacer sequence of single stranded DNA where the displacer
16 sequence is at least partially complementary to such strand of a
17 recipient polydeoxynucleotide sequence. The complex may be
18 stabilized by

- 19
20 a) the presence of at least one modified nucleotide in the
21 displacer strand,
22 b). forming a DNA triplex between the displacer sequence and
23 the recipient duplex,
24 c) providing a displacer strand comprising a nucleotide
25 sequence and a sequence specific DNA binding moiety that does not
26 significantly melt the recipient DNA duplex at the site to which it
27 attaches,
28 d) attaching the displacer to a linker prior to or concurrent
29 with attachment to the target duplex and thereafter covalently
30 attaching the linker to the second strand of the target duplex, or
31 e) a combination of procedures a) and d) or c) and d).
32

33 BRIEF DESCRIPTION OF THE DRAWINGS

34

35 FIGURE 1 (A) depicts a restriction map of pALA-D. R = RsaI, P =
36 PstI. Fragments A - D are labeled above the line, with the

1 nucleotide lengths indicated beneath. There is a single SmaI
2 site in fragment D. (B) Branch migration of displacer (open
3 rectangle), bound to linker (filled rectangle), into a recipient
4 duplex with a four base 3'-overhang (PstI end of fragment
5

6 FIGURE 1 (B) shows the conversion between the duplex bound
7 to the 3' overhang only (left) and following complete branch
8 migration (right).
9

10 FIGURE 1 (C) depicts the maximum displacement with specific
11 pALA-D fragments. m = the maximum number of base pairs
12 which can be formed between the displacer and the
13 complementary recipient strand.
14

15 FIGURE 2 depicts the capture reaction of P-D-BrdC plus P-L-dC.
16 UV fluorogram of 1% agarose gel. Lane 1: RsaI/PstI digested
17 pALA-D (200mg). A, B, C, and D refer to fragments shown in Fig.
18 1. Lanes 2-9: products following ligation in the presence of P-
19 D-BrdC (6 μ g/ml), P-L-dC (2 μ g/ml), and 5 U/ml ligase for 1, 2,
20 4, 8, 16, 32, 64, and 128 min, respectively.
21

22 FIGURE 3 depicts the autoradiogram of Fig. 2. Lanes 1-8
23 correspond to the radiolabeled lanes 2-9 of Fig. 2.
24

25 FIGURE 4(A) depicts an autoradiogram similar to Fig. 3, but with
26 higher ligase concentration and P-D-dC replacing P-D-BrdC.
27

28 FIGURE 4(B) depicts the an early time point in an autoradiogram
29 identical to Fig. 3 except using P-D-BrdC-E(10) replacing P-D-
30 BrdC.
31

32 FIGURE 4(C) depicts an autoradiogram identical to Fig. 3 except
33 using P-D-BrdC-E(24) replacing P-D-BrdC.
34
35
36

FIGURE 5 depicts the autoradiogram of a sequencing gel showing the region of incorporated displacer (bold) and linker (underlined) sequences.

FIGURE 6 depicts BCR using pALAD-G4, a derivative of pMS19 containing a genomic fragment of human ALAD, and displacer-linker duplex S-D-BrdC and S-L-dC was followed by partial digestion with Sau3A1. Lanes 1, 2, 3, 4, 5 and 8: partial digestion products formed at 1, 2, 3, 4, 5 and 8 minutes, respectively. Bands a-k are partial digest bands of the sizes expected: 300, 406, 1538, 1598, 2706, 2731, 2748, 3198, and multiple large bands produced by sites within the vector, respectively.

FIGURE 7 depicts triplex enhanced branch-migration mediated linker capture. Lanes 1-6: pMS19, cut with NciI and Sall, incubated with BT-D-MedC-1, BT-L-dC-1 and T4 DNA ligase as described in the text, for 0, 1, 3, 10, 30 and 120 min. Lane 7: Molecular weight markers of lambda DNA cut with AvaII Lanes 8-13: pMS19, cut with NciI and Sall, incubated with BO-D-MedC-1, BT-L-dC-1 and T4 DNA ligase as described in the text, for 0, 1, 3, 10, 30 and 120 min.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Our process allows initiation and formation of a branch migrated complex without prior stabilization. We have achieved this result by stabilizing the resulting branch migrated complex concurrently with formation or thereafter, or both.

In one embodiment of our invention a displacer, a linker, a displacer-linker duplex and an improved method of attaching a deoxynucleotide displacer sequence to the end of a strand of a target deoxynucleotide duplex utilizing branch migration are utilized to prepare branch migrated complexes. This procedure requires the use of oligo- or

polydeoxynucleotide displacer-linker duplexes consisting of two strands; a displacer strand and a linker strand.

The displacer strand of the displacer-linker duplex contains a sequence of nucleotides at least partially complementary to a linker strand. The displacer strand is capable of hybridizing to the linker strand and has an overhang at its 3', 5' or both ends. The overhang at one end of the displacer strand will comprise a deoxynucleotide sequence at least partially complementary to one strand of a recipient polydeoxynucleotide duplex.

The presence of an incorrect (non-complementary) nucleotide in the portion of the displacer strand at least partially complementary to the duplex limits branch migration beyond the point of the mismatch. Thus, the initial portion of the overhang of the displacer strand must be complementary to the recipient strand and this complementarity to the recipient must extend over a sufficient number of nucleotide bases to allow formation of at least a transient branch migrated complex with the recipient duplex required for the formation. In the transient branch migrated structure the stability of the structure increases as the number of bases increases. There is no minimum number of bases required for the initiation and formation of the branch migrated structure. It is desirable to have at least the first three (3) bases complementary, preferably at least the first five (5) bases complementary to the recipient.

The opposite end of the displacer strand may be blunt with relation to the linker strand after hybridization to it or either the displacer strand or the linker strand may have an overhang. In one embodiment, one of the linker or displacer strands will have an overhang complementary to an overhang resulting from cleavage by a restriction endonuclease.

The linker strand may be of any size provided only that it is of sufficient size to hybridize to the displacer. It is desirable that the linker be in the range of 10-20 bases in length. In a preferred

1 embodiment of our invention, one end of the linker is covalently
2 linked to one strand of the recipient after branch migration occurs.

3
4 The displacer strand may be hybridized to the linker strand prior to
5 or after reaction of the displacer with the recipient.

6
7 As stated above, prior formation of a stable hybrid between our
8 displacers and a recipient polydeoxynucleotide duplex is not required.
9 Any oligo- or polydeoxynucleotide displacer-linker duplex which is
10 capable of initiating branch migration at the end of a recipient
11 polydeoxynucleotide duplex can be used in our process. Once branch
12 migration has been initiated, our techniques of stabilization
13 concurrent with or subsequent to formation of the branch migrated
14 structure will maintain the complex in its branched form.

15
16 Our displacer-linker duplexes are capable of instituting branch
17 migration at any type of end of a recipient polydeoxynucleotide duplex,
18 such as blunt ends or at ends formed by mechanical shearing. In
19 preferred embodiments, our novel duplexes hybridize to and initiate
20 branch migration at a restriction endonuclease cleavage site, most
21 effectively adjacent to a 3' or 5' single stranded extension on a
22 recipient polydeoxynucleotide duplex.

23
24 We have discovered that modifying the nucleotides in the sequence of
25 the displacer which is at last partially complementary to the target
26 increases the stability of the branch migrated complex. Those
27 modifications which increase the association constant with the
28 complementary deoxynucleotide by at least about 20 percent provide
29 useful results. We prefer those modifications which increase the
30 association constant by at least about 70 percent.

31
32 To stabilize the complex concurrent with formation, one or more,
33 preferably at least about 10 percent of the nucleotides in the sequence
34 of the displacer which is at least partially complementary to the
35 target, are modified.
36

1 Specific modifications which we have found to work well in our
2 invention include those selected from 5-halogenated pyrimidine
3 nucleotides, 5-methyldeoxycytidine, diaminopurine deoxynucleotide,
4 ribonucleotides, and 2'-alkylated ribonucleotides. 5-
5 bromodeoxyuridine or 5-methyldeoxycytidine provide the best results.

6
7 Alternatively, the complex may be stabilized after branch migration has
8 occurred. In this embodiment, the linker of the branch migrated
9 complex is covalently attached to one strand of the reagent duplex.
10 Preferably the branch migration complex is incubated with a ligase.
11 The covalently linked linker strand prevents the uncoupling of the
12 branch migrated structure.

13
14 In a preferred embodiment of our invention, the branch migrated
15 structure is stabilized by a combination of concurrent and subsequent
16 stabilization techniques, utilizing modified nucleotides and covalent
17 linkage.

18
19 Our displacer-linker may be modified by incorporation of a label to
20 allow detection of the hybrid construct. Any modification which
21 permits detection and which does not disrupt the branch migrated
22 complex may be used. Common detection modifications are
23 radioactive labels, fluorescent and chemiluminescent labels, enzymes
24 and targets for detection, including, by way of non-limiting example,
25 biotin moieties, phosphorothioate linkages and antigens. For details
26 on the use of various labels and detection systems see, e.g. Keller, G.H.,
27 et al., DNA Probes (1989) and Piper, M.A., et al., Nucleic Acid Probes
28 (1989).

29
30 In another embodiment, our single stranded displacers are not
31 hybridized to a linker strand and are capable of initiating triplex
32 formation at a point other than at the end of a recipient
33 polynucleotide. This class of displacers comprises

34 1. a first sequence which is capable of initiating triple helix
35 formation, and which comprises

36 a) at least six consecutive pyrimidine bases or

1 b) at least seven bases where at least six of the
2 bases are pyrimidine bases and the seventh base is
3 guanine, and
4 2. a second sequence proximate to such first sequence which is
5 a) complementary to and runs antiparallel to the second
6 strand of the recipient duplex and
7 which are capable of initiating branch migration proximate to the
8 triple helix.

9
10 In one embodiment of our invention, the second sequence is adjacent
11 to the first sequence. In another embodiment the second sequence is
12 separated from the first sequence by intervening moieties. In this
13 embodiment, we prefer separation by 1 to 5 intervening moieties.

14
15 Intervening moieties may be selected from any moiety which when
16 interposed between the first and second sequence will not abolish the
17 coordinate function of the two sequences. Examples are intercalating
18 agents and agents that function to diminish the rigidity of the
19 nucleotide sequence, thereby facilitating the hybridization of the
20 complementary sequence to the antiparallel strand. Such moieties
21 include moieties containing sugar phosphate linkages. In a preferred
22 embodiment, the intervening moieties are nucleotides. The
23 nucleotides may be modified nucleotide. Useful modified nucleotides
24 include those having an intercalating agent covalently attached and
25 modified nucleotides incapable of base pairing.

26
27 Once the displacer hybridizes with the antiparallel strand it is
28 desirable to increase the stability of the complex. We have found that
29 we are able to increase the stability by modifying at least one of the
30 nucleotides in the displacer strand prior to hybridization. The
31 nucleotide is modified in a manner that increases the stability of the
32 hybrid displacer-recipient duplex. The modification may be in either
33 the first sequence which is capable of initiating formation of the
34 complex, or in the second sequence proximate to such first sequence
35 and which is complementary to and runs antiparallel to the second
36 strand of the recipient duplex.

1
2 When the modification is in the first sequence it is preferably selected
3 from the group consisting of modified nucleotides which increase the
4 association constant with the complementary deoxynucleotide by at
5 least about 20 percent, preferably at least about 70 percent.

6 Representative non-limiting examples of such modified nucleotides
7 include 5-halogenated pyrimidine nucleotides. Most preferably, the
8 modified nucleotide is selected from the group consisting of 5-
9 bromodeoxyuridine and 5-methyldeoxycytidine.

10
11 When the modification is in the second sequence it may be selected
12 from the group consisting of 5-halogenated pyrimidine nucleotides, 5-
13 methyldeoxycytidine, diaminopurine deoxynucleotide, ribonucleotides
14 and a 2'-alkylated ribonucleotides. Preferably the modified nucleotide
15 is a 5-halogenated pyrimidine nucleotide, especially 5-
16 bromodeoxycytidine, or 5-methyldeoxycytidine.

17
18 In addition to the stability increasing modification, we have found it
19 useful to incorporate modified nucleotides in the displacer which
20 permit detection of the displacer-recipient hybrid. Suitable
21 modifications may be selected from the group comprising radioactive
22 labels, fluorescent and chemiluminescent labels, enzymes and targets
23 for detection, including, by way of non-limiting example, biotin
24 moieties, phosphorothioate linkages and antigens.

25
26 We have also developed displacers which are exonuclease resistant.
27 These nuclease resistant displacers are suitable for use in cell culture
28 and living organisms. These displacers contain at least one moiety
29 conferring exonuclease resistance attached at or near the terminal
30 nucleotide of the displacer.

31
32 The moiety conferring nuclease resistance may be attached to the
33 deoxyribose moiety at the hydroxyl group or the phosphate moiety of a
34 terminal nucleotide.

1 The moiety conferring nuclease resistance may be selected from the
2 group consisting of intercalating agents, isoureas, carbodiimides and
3 N-hydroxybenzotriazoles, polypeptides and proteins.

4
5 A preferred moiety is when the group is attached to the hydroxyl
6 group is a methylthiophosphonate.

7
8 A modified or unmodified 2',3' dideoxyribose nucleotide attached to
9 the 3'-terminal deoxyribonucleotide through a phosphodiester linkage
10 is useful for conferring resistance at the 3' end.

11
12 The formation of complexes utilizing either our single stranded
13 displacer or our displacer-linker proceeds in accordance with well
14 known principles. [Maniatis, et al., (1982) Molecular Cloning (New
15 York: Cold Spring Harbor Laboratories].

16
17 One of the significant uses of our invention is for the site specific
18 addition or deletion of nucleotides in a recipient
19 polydeoxynucleotide sequence. This process occurs when the
20 new strand is introduced to the recipient duplex and displaces
21 the original strand. The cellular machinery involved in
22 generalized recombination and gene conversion will act to
23 transfer sequence information from the displacer strand to the
24 recipient polydeoxynucleotide. For a review of possible
25 mechanisms for this transfer see Stahl, F.W., Genetic
26 Recombination (1979).

27
28 The following Examples are set forth to illustrate various aspects
29 of the present invention but are not intended to limit in any way
30 its scope as more particularly set forth in the claims.

31 32 EXAMPLES

33 34 Example 1

35 36 Preparation of Labeled Displacer and Linker Strands

1
2 The sequences of the oligonucleotides used in this example, as
3 well as in the other examples provided, are listed in Table I. These
4 oligonucleotides were synthesized on an Applied Biosystems Model
5 380B DNA Synthesizer using standard phosphoramidite chemistry, but
6 any other standard method for synthesizing oligonucleotides would
7 work as well for this invention.
8

9 The bromodeoxycytidine (BrdC) and the methyldeoxycytidine
10 (MedC)-containing displacer strands used in these examples were
11 prepared by incorporation of 5-bromodeoxycytidine or 5-methyl
12 deoxycytidine phosphoramidite monomers into the oligonucleotides
13 during their synthesis. Purification steps were limited to hydrolysis of
14 base-protecting groups and cleavage from the support with NH_4OH ,
15 evaporation, resuspension and ethanol precipitation. All
16 oligodeoxynucleotides were 5'-labeled with ^{32}P using T4
17 polynucleotide kinase, subjected to polyacrylamide gel electrophoresis
18 on 20% acrylamide-8M urea gels, and visualized by autoradiography. A
19 single oligodeoxynucleotide species of the correct size was routinely
20 detected.
21

22 To measure capture at recipient ends with 3' or 5' overhangs,
23 linker or displacer oligodeoxynucleotides, respectively, were labeled
24 with ^{32}P using T4 polynucleotide kinase. The reaction of linkers, but
25 not displacers, with labeled ATP was followed by reaction with excess
26 unlabeled ATP to ensure that all linker strands contained a 5'-
27 phosphate. The oligodeoxynucleotides were purified over spun
28 columns of Sephadex G-50 [Maniatis, et al., (1982) Molecular Cloning
29 (New York: Cold Spring Harbor Laboratories).
30

31 Example 2

32 Stability of BrdC-Containing Hybrids

33
34
35 Melting temperatures for oligodeoxynucleotides were
36 determined as described by Quartin & Wetmur [Biochem. 28:1040-47

(1989)]. Briefly, complementary oligodeoxynucleotides were mixed in equal molar ratios in either 6X SSC, pH 4.0, 6X SSC, pH 7.0, or in 1 M NaCl, 0.05 M borate, 0.2 mM EDTA, pH 10, then placed in a 1 cm quartz cuvette. Solutions were heated in the cuvettes at 0.3°C/minute in a Beckman Model 25 spectrophotometer equipped with a water-jacketed cell holder. The solution temperature was monitored using a thermistor attached to the cell holder. Hyperchromicities for all duplexes tested were 21%. The concentration-dependent melting temperatures ($T_m = t_m + 273.16$) were calculated according to the method of Marky and Breslauer [Biopolymers 26: 1601-1620 (1987)]. T_m values reported are reliable to $\pm 1^\circ\text{C}$.

The t_m values for dC-containing oligodeoxynucleotides and for their BrdC and MedC-containing analogs are presented in Table II. The substitution of BrdC or MedC for dC leads to an increase in melting temperature $\Delta T_m = T_m' - T_m$, where T_m and T_m' are the melting temperatures for dC- and BrdC- or MedC-containing oligodeoxynucleotides, respectively.

These results indicate that duplexes containing oligonucleotides with BrdC or MedC are more stable than those with only dC.

The association constant for the modified nucleotide, BrdC or MedC, relative to the association constant for dC, base pairing with complementary dG, may be approximated by

$$K = e^{-\Delta H^\circ / (RT_n^2 N_s)} \quad \text{where}$$

ΔH° = enthalpy for formation of the oligodeoxynucleotide duplex

R = gas constant

T_n = average of T_m and T_m' and

N_s = number of modified nucleotides.

Based on this analysis, the association constant increased about 70-90% for BrdC substitution and about 50-70% for MedC substitution.

Example 3

Displacement By BrdC

A gel migration assay was used to monitor the displacement of 5'-³²P-labeled dC-containing oligodeoxynucleotides from unlabeled complementary oligodeoxynucleotides by their BrdC-containing analogs.

Labeled dC-containing oligodeoxynucleotides were annealed to unlabeled dC-containing complementary strands at room temperature in 1 M NaCl at concentrations of 1 and 3 µg/ml, respectively, and then brought to 4°C. BrdC-containing analogs of the labeled strands (at concentrations ranging from 3 to 400 µg/ml) were incubated with dC-containing duplexes as a function of time at various temperatures. Aliquots taken at each time point were diluted into electrophoresis loading buffer and stored at -70°C prior to electrophoresis. Samples were loaded onto a 20% acrylamide gel in 2.5% ficoll 400, and electrophoresis was performed at 4°C at 400 volts, 5-15 milliamps in 89 mM TrisHCl, 89 mM borate, 1mM EDTA, pH 8 (TBE). The gels were dried and subjected to autoradiography.

The rates at which oligonucleotides containing BrdC displace those containing dC are summarized in Table III. Reactions initiated at blunt ends proceed faster as the temperature increases due to increased breathing at the blunt ends. Displacement reactions where initiation occurred at four base overhangs were more than two orders of magnitude faster than reactions initiated at blunt ends. The stability of a duplex formed between the displacing strand and the overhang of a preformed duplex increased with decreasing temperature. Thus, in the temperature range of 22 to 37°C, the rate of displacement decreases with increasing temperature.

These results demonstrate that oligonucleotides containing BrdC displace the dC analogs in a time- and temperature-dependent reaction, even at blunt-ended recipients.

1 The effect of including a linker strand in displacement reactions
2 was investigated at both 3' and 5' 4-base overhangs with G + C content
3 of 0-100%.

4
5 These results demonstrate that displacement rates using
6 displacer-linker duplexes are at least 8-fold faster than displacement
7 rates using only displacer oligodeoxynucleotides. Based on these
8 results we conclude that the methods described in Examples 7-8 are
9 not restricted by the rate of association of displacer - linker duplexes
10 and recipient duplexes.

11 12 **Example 4**

13 **Hybridization of the Displacer to Target DNA**

14
15
16 The BrdC- or MedC-containing displacer strand is hybridized to
17 the complementary strand of a double-stranded target DNA in the
18 absence of a linker. Linkers complementary to a portion of the target
19 DNA are added subsequent to this hybridization step, if desired.

20
21 The target DNA used in Examples 4-9 was plasmid pALA-D, a
22 pUC9 expression vector containing the cDNA sequence of human
23 delta-aminolevulinate dehydratase (ALA-D) [Wetmur, et al. (1986),
24 Proc. Natl. Acad. Sci., USA 83: 7703-7707].

25
26 pALA-D was digested with RsaI and PstI to yield seven
27 fragments, four of which contain one PstI end with a 3'-overhang and
28 one blunt RsaI fragment (Fig. 1A). The standard displacer sequence,
29 P-D-dC, is complementary to the first 24 nucleotides of the 3'-
30 overhanging strand of the PstI end of restriction fragment B.
31 Initiation of displacer hybridization to the recipient target duplex
32 begins with base pairing of the displacer with the four base overhang
33 of the PstI site. The displacer may replace all or part of the
34 homologous portion of the recipient duplex strand by single-stranded
35 DNA branch migration.

36
The digested pALA-D DNA (20 µg/ml) is mixed with the 34-mer
displacer DNA (6 µg/ml) in 25% reduced T4 DNA ligase buffer [50mM

1 Tris-HCl, pH 7.0, 1mM ATP, 10 mM MgCl₂, 20 mM Dithiothreitol
2 (DTT), 50 µg/ml bovine serum albumin (BSA)], incubated at 55°C for
3 10 min., then cooled to room temperature.
4

5 Fig. 1C shows the potential for branch migration of the displacer
6 into pALA-D fragments with one Pst I and one blunt (RsaI) end. For
7 fragment B, where there is full complementarity to the displacer, the
8 maximum number of displacer-recipient base pairs (m) is 24. The
9 maximum number of branch migration steps is (m-4) because four
10 base pairs are formed between the displacer and the 3' PstI overhang.
11 Fragment D and the smallest (71 base pair) PstI-RsaI fragments
12 contain no complementarity beyond the PstI site. Thus, m=5,
13 including the four base overhang and one branch migration step.
14 Fragment A contains four additional nucleotides adjacent to the PstI
15 site which are complementary to the displacer. Thus, m=9, including
16 the four overhang and five branch migration steps. Unlike fragment B,
17 where branch migration may go to completion, all branch migration
18 structures with fragments A and D contain two single-stranded
19 branches.
20

21 Example 5

22 23 Hybridization of the Displacer to Target DNA in the Presence of the 24 Linker 25

26 In a second embodiment of this invention, branch migration-
27 mediated capture of linker oligodeoxynucleotides was performed
28 according to the scheme in Fig. 1 B. In this embodiment, the
29 recipient target duplex, the displacer oligodeoxynucleotides, and the
30 linker deoxyoligonucleotides are all present during the hybridization
31 reaction. The linker sequence, 5'-³²P-labeled P-L-dC, forms a duplex
32 with the 5' portion of the displacer sequence which lacks homology to
33 the recipient duplex. The displacer-linker duplex formed displaces
34 the homologous target strand by base pairing to the recipient target
35 strand.
36

1 pALA-D was digested with PstI and RsaI. Digested plasmid (20
2 µg/ml) was mixed with a 34-mer displacer oligodeoxynucleotide (6
3 µg/ml) and a 5'-³²P-labeled 14-mer linker oligodeoxynucleotide (2
4 µg/ml) in a pH-reduced T4 DNA ligase buffer [50mM Tris-HCl, pH 7.0,
5 1mM ATP, 10 mM MgCl₂, 20 mM Dithiothreitol (DTT), 50 µg/ml
6 bovine serum albumin (BSA)], incubated at 55°C for 10 min., then
7 cooled to room temperature.

8 9 **Example 6**

10 11 **Formation of the Displacer-Linker Duplex**

12
13 In another embodiment of this invention, the initial step of the
14 displacement reaction involves the formation of a displacer-linker
15 duplex and the subsequent addition of this displacer-linker duplex to a
16 target duplex.

17
18 Formation of the displacer-linker duplex may be accomplished
19 in the following way. A 34-mer displacer oligodeoxynucleotide (6
20 µg/ml) is mixed with 5'-³²P-labeled 14-mer linker
21 oligodeoxynucleotide (2 µg/ml) in a pH-reduced T4 DNA ligase buffer
22 [50mM Tris-HCl, pH 7.0, 1mM ATP, 10 mM MgCl₂, 20 mM
23 Dithiothreitol (DTT), 50 µg/ml bovine serum albumin (BSA)],
24 incubated at 55°C for 10 min., then cooled to room temperature.

25 26 **Example 7**

27 28 **Hybridization of the Displacer-Linker Duplex to the Target DNA**

29
30 pALA-D is digested with PstI and RsaI to yield 7 restriction
31 fragments. The digested pALA-D DNA (40 µg/ml) in 50 mM Tris-HCl,
32 pH 7.0, 1mM ATP, 10 mM MgCl₂, 20 mM DTT, 50 µg/ml BSA is
33 mixed with an equal volume of the displacer-linker duplex prepared in
34 Example 6. This mixture is incubated at 55°C for 10 min., then
35 cooled to room temperature.
36

Example 8

Ligation of the Linker Oligodeoxynucleotide to the Target Strand

The efficiency and the specificity of the branch mediated linker ligation reaction is demonstrated in Figures 2 and 3.

The time requirements for the ligation of the pALA-D PstI-RsaI fragments in the presence of the displacer P-D-BrdC (6 $\mu\text{g/ml}$) and the linker P-L-dC (2 $\mu\text{g/ml}$) were established using T4 DNA ligase (5 U/ml ligase) and incubation times ranging from 1-128 min. The results are illustrated in Fig. 3, lanes 2-9. Ligation results in a small, time dependent decrease in the mobility of fragment B. The ligation of the linker to the branch migrated structure is 50% completed in 32 min. and 100% completed within approximately 1 hr. A similar experiment carried out using 25 U/ml ligase proceeded 50% to completion in 8 min. Thus, the reaction rate for this ligation reaction was linearly dependent on ligase concentration and time. These results show that branch mediated ligation of a linker oligonucleotide is an efficient reaction that occurs fairly rapidly.

Fig. 3 is an autoradiogram of the gel shown in Fig. 2 and illustrates the detection of ligation of 5'- ^{32}P -labeled linkers to the recipient fragments. Under conditions where the reaction with fragment B ($m=24$) is carried to completion, fragment A ($m=9$) is barely visible and fragment D ($m=5$) is undetectable. The majority of the labeled linkers are associated with fragment B. These results demonstrate the specificity of branch mediated linker capture.

Example 9

Specificity of the Branch Migration Reaction

The requirement of BrdC- or MedC-containing displacer strands for specific and efficient branch migration-mediated linker capture is illustrated in the following experiment. Fig. 4A shows an

1 autoradiogram analogous to that presented in Fig. 3, where the
2 displacer strand is P-D-dC instead of P-D-BrdC. Under conditions
3 where the reaction with fragment B is carried 50% to completion,
4 both fragment A and Fragment D were easily detected. Table IV
5 contains relative autoradiographic intensities for fragments A, B and D,
6 using P-D-BrdC, P-D-MedC and P-D-dC as displacer molecules. The
7 intensity of fragment D was assigned the value of 1. Since the
8 intensities for fragments A and B were determined using a 2-fold time
9 course assay, they are reliable to +/-50%. The data in Table IV
10 demonstrate that the use of BrdC or MedC in the displacer is
11 important for obtaining highly specific branch migration-mediated
12 capture.

13
14 Displacer P-D-BrdC-B(10) was synthesized with one incorrect
15 nucleotide such that only 9 base pairs could be formed between the
16 displacer and the complementary recipient strand prior to the
17 mismatch. Fig. 4B is an autoradiogram of a branch migration-mediated
18 capture reaction carried out under conditions leading to trace levels of
19 capture. The intensity ratios are given in Table V. The capture by
20 fragment B (m=24 with mismatch at 10) was exactly the same as that
21 of fragment A (m=9). Thus, a single mismatch at position 10 blocked
22 subsequent branch migration, indicating that the branch migration
23 reaction is extremely specific.

24
25 Fig. 4C shows an autoradiogram analogous to that in Fig. 3, where
26 the displacer was P-D-BrdC-B(24). The final nucleotide of P-D-BrdC-
27 B(24) differed from that of P-D-BrdC. The results were similar to
28 those in Fig. 3. Thus, the formation of the final singly-branched
29 structure (Fig. 1B, lower right) is unnecessary to achieve the high
30 specificity observed for capture reactions of BrdC-containing
31 oligodeoxynucleotides.

32
33 Experimental and theoretical intensity ratios are compared in
34 Tables IV and V. The theoretical intensity ratios were calculated
35 using:
36

$$(1) \quad r_i / r_j = (q_i - 1) \cdot q_j / (q_j - 1) \cdot q_i$$

The partition functions, q_i and q_j , are close to 1 if the recipient ends are not saturated with displacer-linker duplexes. Then:

$$(2) \quad r_1 / r_2 (m=5) = (1 + \sum_{k=5}^m \pi B_k + \Omega \sum_{i=5}^{m-1} \pi B_k) / (1 + \Omega)$$

$$(3) \quad r_2 (\text{all } m) / r_2 (m=5) = (1 + \Omega \sum_{i=5}^m \pi B_k) / (1 + \Omega)$$

Equation (2) was used for complete branch migration (Fragment B with displacer P-D-BrdC, P-D, MedC or P-D-dC). Equation (3) applied to all branch migration terminated by a mismatch at position $M + 1$. For these calculations, $\Omega > 2$, $B_k = 1.7 - 1.9$ for BrdC and $B_k = 1$ for dC.

B_k is the relative association constant for base pair k . B_k is usually 1. For BrdC, $B_k = 1.7 - 1.9$ as determined in Example 2. Similarly for MedC, $B_k = 1.5 - 1.7$. The excellent qualitative agreement between theory and experiment suggests that the theory may be used for the design of displacer molecules.

Example 10

Linker Capture at 5' Ends

For capture at a 5' overhang, the directions of the displacer and linker strands were reversed, with the linker forming a duplex with the 3' portion of the displacer sequence which lacks homology to the recipient duplex.

Plasmid pALA-D-G3 is a pUC19 clone containing a 3.2 kb genomic fragment of human ALA-D. Oligodeoxynucleotides E-D-BrdC and X-D-BrdC were synthesized as displacers for branch migration at the unique EcoRI and XmaI, respectively. The same linker, E-L-dC (X-L-dC) was employed for both displacers.

pALA-D-G3 was digested with either EcoRI or XmaI and a second restriction endonuclease to produce convenient sizes of

1 recipient fragments. This product was mixed with PstI plus RsaI
2 digested pALA-D (Example 4) and capture reactions were carried out
3 with equimolar P-L-dC and E-L-dC and 400 nM P-D-BrdC and either
4 E-D-BrdC or X-D-BrdC.

5
6 The linker capture reactions were performed as described in
7 Example 8, using 25 U/mL T4 DNA ligase, and analyzed using the gel-
8 shift analysis illustrated in Figure 2. The half-time for linker capture
9 was 5' at EcoRI (0% G+C 5' overhang) and 20' at XmaI (100% G+C 5'
10 overhang) when the half-time for linker capture in the same reaction
11 5' for PstI (50% G+C 3' overhang). Thus, the linker capture method
12 may be applied to either 5' or 3' overhangs and overhangs of all
13 possible G+C percent.

14
15 pALA-D was cut with SmaI, which produces blunt ends, and a
16 second enzyme, and a capture reaction was carried out using X-D-BrdC
17 and Sm-L-dC. Linker capture was observed, although the rate was
18 more than two orders of magnitude slower than linker capture at
19 recipient ends with 3' or 5' overhangs.

20 21 **Example 11**

22 23 **Selective Cloning of Branch Migration Structures**

24
25 The product of a branch migration-mediated capture reaction
26 similar to those described in the previous examples was ligated into a
27 vector and used to transform E. coli using standard cloning
28 techniques, such as those described by Maniatis, et al. (1982).

29
30 The plasmid, pALA-D, was digested with PstI leading to 2771
31 and 1037 nt fragments. The 2771 pUC9-containing fragment was
32 inactivated as a cloning vector by digestion with SmaI, which leads to
33 two blunt-ended fragments. Each end of the 1037 nt fragment
34 terminated in a Pst I site, one of which was the same as the Pst I site
35 in fragment B. The branch migration-mediated capture reaction was
36 carried out as described in Examples 7 and 8 using P-D-BrdC and P-L-

1 dC. The products were ligated into pUC19 digested with SphI and
2 PstI, both of which cut in the polylinker region of the vector. The
3 SphI end contained a 3'-overhang complementary to the overhang
4 created by the capture of the displacer-linker duplex.
5

6 Four independent plasmid clones were digested by various
7 restriction endonucleases and shown to have the patterns expected
8 for linker incorporation following electrophoretic analysis of the
9 resulting restriction digestion products. One of these clones was
10 selected for further analysis using standard double-stranded DNA
11 sequencing techniques. The autoradiogram of the sequencing gel
12 depicted in Fig. 5 confirms the identity of the cloned insert as being
13 the product resulting from branch migration-mediated capture and
14 linker ligation. These results suggest that *E. coli* contain polymerases
15 and nucleases capable of handling both BrdC substitutions and
16 branched structures.
17

18 Thus, the branch migration product obtained according to the
19 methods of this invention can be cloned directly using standard
20 cloning techniques. Neither the presence of BrdC, nor the presence
21 of a branched structure interfered with the cloning process.
22

23 Example 12

24 **Branch migration mediated labeling and partial digest mapping.**

25 The plasmid pMS19 was constructed by inserting PMS-ES/PMS-
26 SE and PMS-NH/PMS-HN into the EcoRI and HindIII sites,
27 respectively, of pUC19 in the order indicated in Table I. The
28 insertions destroyed the EcoRI and HindIII sites adjacent to the
29 plasmid and inserted adjacent BglII (AGATCT) sites at each end of the
30 expanded linker region. The new polylinker contains an SfiI site, a
31 branch migration target sequence, the entire polylinker of pUC19, a
32 different branch migration target sequence, and a NotI site.
33
34
35
36

Using the BglIII sites, this entire region expanded polylinker has been cloned into unique BamHI sites in other vectors including a cosmid vector. The reading frame was maintained so that pMS19 produces blue colonies when grown with IPTG and X-gel. A 3.6 kb EcoRI-HindIII genomic fragment of human ALA-D, the sequence of which had previously been determined, was cloned into the polylinker of pMS19 to produce the plasmid pMS19-G4.

pMS19-G4 was digested with NotI and SfiI. The product was end-labeled by branch migration mediated capture of 32 P-labeled linker (SfiI) or displacer (NotI) using S-D-BrdC and S-L-dC to label the SfiI site or N-D-BrdC and N-L-dC to label the NotI site. This product was digested for various times with Sau3A1 and the products were analyzed by agarose gel electrophoresis. An autoradiogram of mapping at the SfiI end of the cloned fragment is depicted in Figure 6. The resultant Sau3A1 map agreed with the known DNA sequence.

Because the melting temperature of the linker-displacer duplex is not critical for the branch migration process, labeling is not limited to radioactive labels, but may be extended to include a wide variety of labels permitting either detection or affinity purification of a labeled fragment. Thus, specific branch migration mediated labeling of restriction fragments may be applied to a wide variety of mapping and isolation techniques.

Example 13

Formation of Triple Helices

The target DNA used in Examples 13-14 was plasmid pMS19, described in Example 12.

Superhelical pMS19 DNA was mixed with an equal quantity of pMS19 which had been linearized by cleavage with EcoRI. Aliquots of this DNA mixture (20 μ g/ml) were mixed with either 32 P-labeled BT-D-MedC-1 (1 μ g/ml) or 32 P-labeled BO-D-MedC-1 (1 μ g/ml)

1 oligodeoxynucleotide in 100 mM sodium citrate, pH 4.8, at room
2 temperature and allowed to cool to 4°C. BT-D-MedC-1 and BO-D-
3 MedC-1 both contained sequences capable of forming a branch-
4 migrated structure, whereas only BT-D-MedC-1 contained the
5 polypyrimidine sequence capable of participating in triple helix
6 formation.

7
8 The products of the incubation were separated by
9 electrophoresis at 4°C in a 1.2% agarose gel in 100 mM sodium
10 citrate, pH 4.8. The gel was dried for autoradiography. Both the
11 superhelical and the linear forms of pMS19 stably bound ³²P-labeled
12 BT-D-MedC-1. Neither the superhelical nor the linear form stably
13 bound ³²P-labeled BO-D-MedC-1.

14
15 Thus, the ability to form a branch-migration product, even at a
16 low pH where branch migration is most favored, was not sufficient for
17 forming a stable complex with linear or even with superhelical DNA.
18 On the other hand, a stable triple-helical product was formed with
19 both linear and superhelical DNA when the polypyrimidine sequence
20 was present.

21 22 **Example 14**

23 24 **Coupling of branch migration with triple helix formation.**

25
26 The recipient plasmid was pMS19 cut with NciI and SalI. SalI is
27 an enzyme producing a 5' overhang. Displacer molecules were either
28 ³²P-labeled BO-D-MedC-1 or ³²P-labeled BT-D-MedC-1. The linker
29 molecule was BT-L-dC-1. Displacer-linker duplexes were formed as
30 described in Example 6, hybridized as described in Example 7 and
31 ligated as described in Example 8 except that the concentration of
32 displacer-linker duplex was 0.2 μM, the solvent was 25 mM tris-
33 acetate, pH 6.8, 10 mM NaCl, 1 mM spermine, 1 mM DTT, 10 mM
34 MgCl₂, 1 mM ATP.